EXHIBIT 6

Phase I Pharmacokinetic and Pharmacodynamic Study of Recombinant Human Endostatin in Patients With Advanced Solid Tumors

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Purpose: **Endostatin is the first endogenous angiogenesis inhibitor to enter clinical trials. Laboratory investigations with endostatin have indicated broad antitumor activity coupled with remarkably low toxicity. A phase I trial of recombinant human endostatin was designed to evaluate toxicity and explore biologic effectiveness in patients with refractory solid tumors.**

Patients and Methods: **Endostatin was administered as a 1-hour intravenous infusion given daily for a 28-day cycle. A starting dose of 30 mg/m^2 was explored with subsequent dose escalations of 60, 100, 150, 225, and 300 mg/m^2. Assessment of serum pharmacokinetics was performed on all 21 patients. Western blot assay and mass spectroscopy were employed to evaluate endostatin metabolism. Circulating levels of endogenous proangiogenic growth factors were examined. Tumor and tumor blood supply were imaged by dynamic computed tomography (CT), magnetic resonance imaging, ultrasound, and positron emission tomography.**

Results: **Endostatin given on this schedule was essentially free of significant drug-related toxicity. Two transient episodes of grade 1 rash were observed. No clinical responses were observed. Endostatin pharmacokinetics were linear with dose, and serum concentrations were achieved that are associated with antitumor activity in preclinical models. No aggregate effect on circulating proangiogenic growth factors were seen, although several patients exhibited persistent declines in vascular endothelial growth factor levels while enrolled in the study. A few patients demonstrated changes in their dynamic CT scans suggestive of a decline in microvessel density, although overall, no consistent effect of endostatin on tumor vasculature was seen.**

Conclusion: **Endostatin given daily as a 1-hour intravenous infusion was well tolerated without dose-limiting toxicity at doses up to 300 mg/m^2.**

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ANGIOGENESIS, THE formation of new capillaries from existing vasculature, is a process fundamental to the development of solid tumors.[1-5] Tumors that are unable to elicit angiogenesis exist in a dormant state, and are unable to grow beyond a few millimeters in size. This complex, multistep process of angiogenesis involves extracellular matrix degradation, endothelial cell proliferation, and migration, followed by formation of the vascular lumen. A myriad of potential therapeutic targets exist in this pathway. Angiogenesis is fundamental to wound repair, reproduction, and development. It is not surprising, therefore, that this process is tightly regulated, and is subject to a large number of positive and negative regulatory factors. The central role of angiogenesis in tumor growth has fostered an intense search for agents that might inhibit this process. One strategy has been to inhibit proangiogenic signal transduction. A large number of endogenous proteins that act to promote normal and/or tumor angiogenesis have been described including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and many others.[5-8] Therapeutic interventions currently in clinical trials include antibodies against VEGF and small molecule VEGF receptor tyrosine kinase inhibitor. An alternative approach has been to increase the levels of naturally occurring antiangiogenic compounds. Endostatin represents the first of these factors to enter into human clinical trials.

Endostatin is a naturally occurring peptide originally isolated from a murine hemangioendothelioma cell line.[9,10] O'Reilly et al[10,11] purified endostatin from conditioned media by analyzing in vitro inhibition of capillary endothelial cell proliferation. Amino acid analysis of this 20-kDa peptide has shown it to be identical to the noncollagenous (NC1) carboxy terminal end of collagen XVIII. Both the purified peptide and recombinant endostatin inhibited endothelial cell proliferation in vitro. Endostatin also inhibited in vivo angiogenesis when the chick chorioallantoic membrane assay was used.[10] O'Reilly et al further demonstrated that systemically administered endostatin inhibited the growth of Lewis lung metastases and could also inhibit a number of primary tumors including Lewis lung, T241 fibrosarcomas, and B16F10 melanomas. In established tumors, endostatin induced tumor regressions by inhibiting angiogenesis and increasing tumor cell apoptosis.[10] In subsequent investigations, this group has also shown that repeated treatment with endostatin does not induce in vivo resistance to its antitumor effect.[12] In some instances, prolonged dormancy could be induced even after discontinuation of endostatin administration.

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Since the discovery of endostatin in 1996, a large number of preclinical studies have been performed with this compound.[10,12-30] The ability of endostatin to inhibit nonxenograph tumor models has been demonstrated in both carcinogen-induced mammary tumors[20] and in transgenic mice.[17] Improved tumor inhibition has been seen when endostatin is combined with radiation[31] or angiostatin.[32,33]

Elevated circulating endogenous serum endostatin concentrations have been measured in patients with a number of different cancers.[34-39] Increases in serum endostatin have also been seen in patients with inflammatory conditions such as rheumatoid arthritis[40] and systemic sclerosis.[41] The regulation of proteolytic release of endostatin from collagen XVIII is being actively investigated. Both elastase[42] and secreted cathepsin L[43] can generate endostatin from collagen XVIII.[44]

The mechanism by which endostatin exerts its pleiotropic effects on neovasculature endothelial cells is unknown. Endostatin can inhibit growth-factor–induced signal transduction.[25,45,46] An Src homology 2 domain contacting adaptor protein B was found to mediate endostatin-induced apoptosis.[19] Kim et al[16] demonstrated that endostatin can block the activation and catalytic activity of matrix metalloproteinases. Recently, endostatin has been shown to interact with the cytoskeletal protein tropomyosin.[47] Tropomyosin protein fragments also inhibited the antiangiogenic effect of endostatin.[47]

Extensive toxicologic evaluation of recombinant human endostatin has been performed. Endostatin, given subcutaneously or by intravenous infusion, exhibited essentially no toxicity in a range of doses from 5 to 100 mg/kg in mice or cynomolgus monkeys.[48] Low titers of immunoglobulin G antiendostatin antibodies were detected after 14 days of treatment. These antibodies did not result in a clinical reaction and did not affect the serum clearance. Berger et al showed that endostatin does not inhibit physiologic angiogenesis that occurs with wound healing.[49] Friedl et al, however, found an inhibition in blood vessel maturation with endostatin.[26]

Phase I clinical trials of endostatin given as multiple daily injections were initiated at the University of Wisconsin, the M.D. Anderson Cancer Center, and the Dana-Farber Cancer Institute. Intravenous administration was chosen over the subcutaneous route because of the volume needed with the available endostatin formulation. In our trial, endostatin was given as a daily 1-hour intravenous infusion on a 28-day cycle. A number of histopathologic and radiologic pharmacodynamic evaluations were performed in this trial and are the subject of separate articles.

PATIENTS AND METHODS

Patient Selection

The study was approved by the Human Subjects Committee at the University of Wisconsin, which functions under a multiple project assurance issued by the Department of Health and Human Services. Adult patients (> 18 years old) with histologically confirmed solid tumors refractory to standard therapy (or for which no standard therapy existed) were eligible for the study. The patient must have had tumors amenable to serial core biopsies. An Eastern Clinical Oncology Group performance status of 0 or 1 was required. The patient could not have received any previous chemotherapy or radiotherapy for 4 weeks before study entry. Patients with primary brain tumors or brain metastases were not eligible. No serious active infections were allowed and pregnant or lactating women were excluded. Patients were required to provide signed, written informed consent. Adequate organ function was required including hematopoietic (white blood cell count > 4,000 mm^3 or absolute neutrophil count > 2,000 mm^3, platelets > 100,000), hepatic (bilirubin within normal limits, AST < two times the upper limit of normal) and renal (serum creatinine < 1.5 mg/dL or calculated creatinine clearance of > 60 mL/min).

Treatment Plan

This phase I trial was designed as an open-label, nonrandomized dose escalation study in which groups of three to six patients were to receive sequentially increasing dosages of intravenous recombinant human endostatin until dose-limiting toxicity (DLT) was seen in at least two of six patients. The maximum-tolerated dose was defined as the dose level at which zero of six or one of six patients experience DLT, with the next higher doses having at least two patients encountering DLT. Dose levels beyond 300 mg/m^2 were not planned because of formulation and drug supply issues. Endostatin was administered daily for 28 days followed by a 7-day break to evaluate cumulative toxicity. Subsequent 28-day cycles proceeded without an intervening break. Given the lack of preclinical toxicity, a starting dose of 30 mg/m^2 was determined empirically after discussion with the sponsor and the National Cancer Institute (NCI). This represented one tenth of the planned maximal dose.

DLT was defined as the occurrence of grade 4 hematologic toxicity or grade 3 or 4 nonhematologic toxicity. Patients with grade 3 or 4 treatment-related toxicities were required to recover to at least grade 1 before they could resume treatment at the next lower dose level. Patients with ≥ 50% disease progression were removed from the study. Patients with progression less than 50% were allowed to remain in the study if no other active therapeutic options were available to the patient and the patient required no palliative interventions.

Drug Supply

Recombinant human endostatin (L99–0046, NSC 704805) was supplied by the NCI in sterile, single-use vials containing 8 mg/mL of rhendostatin in a buffer containing 17 mmol/L citric acid, 66 mmol/L sodium phosphate, and 59 mmol/L sodium chloride, pH 6.2. Vials of citrate buffer were also provided by the NCI. The 8 mg/mL endostatin solution was further diluted with the buffer solution to produce an infusion volume of 75 mL. Patients whose endostatin dose volume alone exceeded 75 mL were administered undiluted endostatin.

Pharmacokinetics

Serum endostatin levels were assessed for all patients using a polyclonal enzyme immunoassay (EIA) 96-well plate assay (Accucyte, Cytimmune Sciences Inc., College Park, MD). The assay was validated using purified recombinant human endostatin as a standard. This assay had a detection limit of 2 to 500 ng/mL, a mean coefficient of variance of 6.4%, with a range of 5.1% for the high standard (125 ng/mL) to 8.1% for the lowest standard (3.9 ng/mL). In cycle 1, blood samples on day 1 were drawn preinfusion, at the end of the 1-hour infusion, and then 0.5, 1, 2, 4, 6, 8, 12, and 24 hours postinfusion. Blood samples were also drawn before and at the end of infusion on days 2, 5, 8, 15, 22, and 28. In subsequent cycles, blood was drawn on days 1 and 15. After centrifugation, serum aliquots were prepared and stored at −70°C until analysis. No loss of immunoreactive endostatin was seen with a limited number of freeze-thaw cycles. All postinfusion endostatin serum concentrations were corrected for the predrug endogenous endostatin concentration on day 1. Dose 1 concentration-time data sets were examined by both compartmental and noncompartmental methods using PKAnalyst (MicroMath Research, Salt Lake City, UT). Pharmacokinetic parameters were calculated by standard methods. The dose 1 area under the curve (AUC) was determined by the linear trapezoidal rule, extrapolated to infinity. Dose 1 total clearance (Cl_t) was determined by (dose)/(extrapolated AUC).

Mass Spectroscopy

The metabolic fate of endostatin was examined in a limited number of samples. Patient serum was diluted 1:1 with citrate phosphate buffer (pH 6.2)

Table 1. Endostatin Pharmacokinetic Parameters (mean ± SD)

Daily Dose (mg/m^2)	Day 1 Pre ($\mu g/mL$)	Day 1 C_{max} ($\mu g/mL$)	Day 1 AUC ($\mu g/mL \times hour$)	Day 1 Cl_t (L/h)	$t_{1/2}\ \beta$ (hour)	Day 28 C_{min} ($\mu g/mL$)	Day 28 C_{max} ($\mu g/mL$)
30 (n = 3)	0.035 ± 0.024	0.578 ± 0.099	1.92 ± 0.56	36.6 ± 16.5	7.4 ± 4.0	0.075 ± 0.032	0.589 ± 0.022
60 (n = 6)	0.022 ± 0.008	1.18 ± 0.169	2.74 ± 0.61	47.2 ± 14.4	13.4 ± 4.9	0.129 ± 0.057	1.57 ± 0.525
100 (n = 3)	0.025 ± 0.011	2.47 ± 0.584	5.90 ± 2.82	39.3 ± 19.5	13.7 ± 1.4	0.220 ± 0.173	2.75 ± 0.967
150 (n = 3)	0.025 ± 0.017	3.26 ± 0.780	7.39 ± 1.80	37.4 ± 6.3	8.2 ± 1.3	0.273 ± 0.098	4.45 ± 1.05
225 (n = 3)	0.023 ± 0.004	6.20 ± 1.38	14.4 ± 1.21	31.6 ± 4.1	16.5 ± 0.9	0.527 ± 0.237	7.03 ± 1.36
300 (n = 3)	0.041 ± 0.003	9.71 ± 0.83	24.5 ± 2.40	26.5 ± 7.0	18.1 ± 5.9	0.947 ± 0.852	11.22 ± 1.66
Overall	0.028 ± 0.013	NA	NA	37.9 ± 13.4	12.9 ± 5.1	NA	NA

Abbreviation: NA, not applicable.

and then fractionated by fast protein liquid chromatography (FPLC) (Sephadex 75, 0.5 mL injected, 0.5 mL/min; Pharmacia LKB, Uppsala, Sweden) and proteins lower than 25 kDa were then concentrated with a filter (Millipore/Amicon, Bedford, MA). EIA evaluation demonstrated near-complete recovery of endostatin in the low molecular weight pool. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (12.5%) and analyzed by Western blot assay using a polyclonal antiendostatin antibody. Lower molecular weight fractions were then applied to a triple quadrupole electrospray ionization mass spectrometer (API 365, Perkin Elmer Sciex, Norwalk, CT) after further separation by reversed-phase high-performance liquid chromatography (HPLC; Vydac C4 column [Vydac, Hesperia, CA], 99% H_2O/0.05% tetrahydrofuran to 90% acetonitrile/0.05% tetrahydrofuran over 50 minutes).

Growth Factor Studies

Plasma VEGF was measured using a 96-well plate sandwich enzyme-linked immunoassay (Quantikine human VEGF, R&D Systems, Minneapolis, MN). Sensitivity of this assay was 9 pg/mL, with a linear standard curve from 30 to 2,000 pg/mL. bFGF in plasma was measured using a sandwich enzyme-linked immunoassay (Quantikine HS human FGF basic, R&D Systems). Sensitivity of this assay was 0.3 pg/mL in plasma.

Imaging

Imaging was obtained before treatment initiation and then at 4 and 8 weeks.

Dynamic computed tomography (CT). One hundred milliliters of omnipaque was administered at a rate of 5 mL/sec intravenously. Images were obtained with a Lightspeed multirow helical scanner (one slice/3 sec; GE). Regions of interest in the tumor periphery were compared to the aorta and time versus attenuation curves were generated.

Dynamic magnetic resonance imaging (MRI). Gadolinium diethylenetriamine-pentaacetic acid (Gd-DTPA) was administered intravenously and images obtained with a 1.5-Tesla system (Signa LX, GE Medical, Milwaukee, WI). Tumor enhancement was assessed with a fast, spoiled gradient recall echo approach and tumor signal intensity over time analyzed with Functool software (GE Medical Systems).

Positron emission tomography (PET). PET images were obtained with an Advance PET Imaging System (GE Medical Systems) 45 minutes after injection of 10 mCi of F-18-fluorodeoxyglucose.

Ultrasound. Tumors were imaged with gray scale, color velocity, and color energy ultrasound.

RESULTS

Patient Characteristics

Twenty-one patients with advanced solid tumors were treated with recombinant human endostatin. The median age was 53.3 years (range 29 to 70). There were 13 men and 8 women enrolled. The cancers treated included colon,[5] sarcoma,[4] lung,[3] melanoma,[2] and one each of renal, ovarian, adrenal, head and neck, appendiceal, prostate, and gastric. All patients had metastatic disease and had received prior chemotherapy.

Toxicities

Endostatin given as a daily 1-hour intravenous infusion was remarkably well tolerated. There were no DLTs seen. The only drug-related toxicities observed were in two patients with transient grade 1 rashes. Although no drug-related DLTs were seen, the 300 mg/m^2 dose level was the predetermined maximal dose evaluated. This maximal dose level was selected because of drug production issues, and it was believed to be significantly higher than doses associated with preclinical efficacy.

Pharmacokinetics

Endostatin pharmacokinetic assays were performed on all patients (as discussed in Methods) and pharmacokinetic parameters are shown in Table 1. Pre-existing immunoreactive endostatin was detectable in all patients with a mean of 27.6 ± 12.9 ng/mL (mean ± SD; range, 8.2 to 54.2). Pharmacokinetic parameters were corrected for this endogenous endostatin by subtracting the values before day 1. The end of infusion concentrations (C_{max}) of endostatin achieved are shown in Fig 1. C_{max} increased linearly with dose. Peak concentrations achieved ranged from 577.8 ng/mL at the 30 mg/m^2 dose level to 9,712.2 ng/mL at the 300 mg/m^2 dose level. Endostatin AUC versus dose is shown in Fig 2. Endostatin AUC increased linearly with dose. Both the peak and AUC values obtained by the EIA assay at the higher dose levels represent endostatin levels associated with antitumor activity in preclinical models. Endostatin clearance did not vary significantly with dose administered or body-surface area. Endostatin decay was fitted with a biexponential decay model that gave an alpha half-life ($t_{1/2}$) of 42.3 minutes and a beta $t_{1/2}$ of 12.9 hours. In Fig 3, endostatin concentration versus time is shown for a representative patient. With the long terminal $t_{1/2}$, there was some accumulation in the 28-day trough concentration. This increase was also dose dependent, as shown in Fig 4. At the 300 mg/m^2 dose level, the trough concentration of 947 ng/mL represents an approximate 23-fold increase over the baseline endogenous endostatin levels. However, there were no significant increases in peak endostatin concentrations in patients who received two or more cycles. Intraindividual pharmacokinetic variability was minimal. In Fig 5, serum endostatin levels for a single patient over an 8-month period are presented. Antibodies to endostatin were observed in several patients, and have been described by Fogler et al.[50] Antibody formation was not associated with changes in pharmacokinetics, nor was it associated with any clinical symptoms.

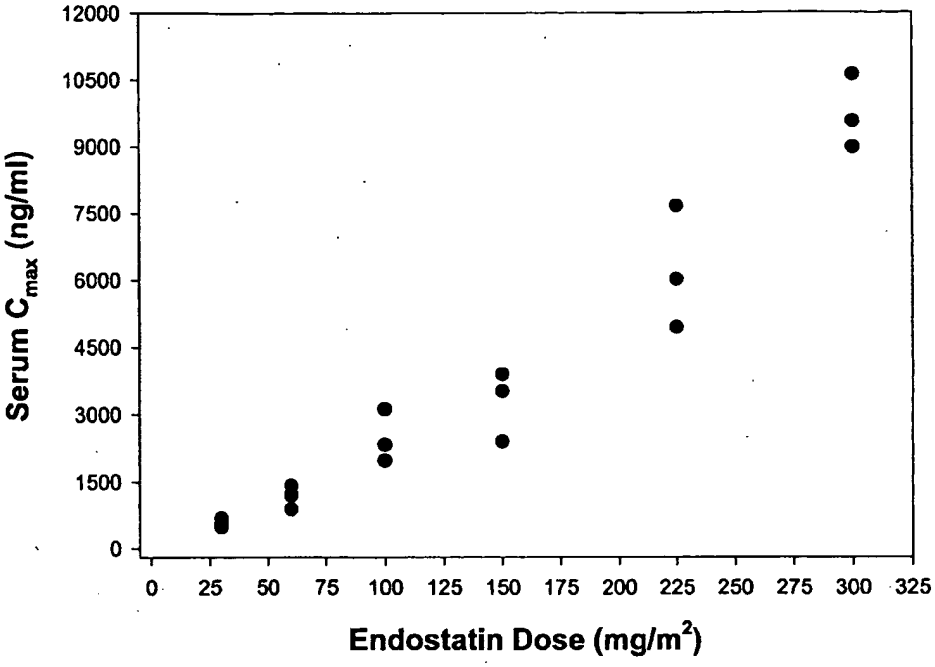

Fig 1. Maximal serum concentration (C_{max}) of endostatin at the end of the first endostatin infusion (day 1 dose) as a function of the endostatin dose level.

We have attempted to further characterize circulating endostatin at various times after the 1-hour infusion. The polyclonal EIA assay noted above gives no information about further proteolytic processing of endostatin in the circulation or about whether it retains biologic activity. We first examined the fate of the administered endostatin by Western blot assay (Fig 6). Lower molecular weight serum proteins were first separated from the large quantity of albumin and immunoglobulins by FPLC. The pooled, less than 25-kDa fractions were then concentrated and subjected to sodium dodecyl sulfate-PAGE followed by Western blot assay using a polyclonal antihuman endostatin antibody. Only a single broad band was seen at the end of the infusion. Total endostatin levels decayed over time, but at 6 hours no immunoreactive lower molecular bands were detected. We further characterized the FPLC fractions by HPLC-mass spectoscopy (Fig 7). The clinical grade endostatin produced predominantly two bands of 19.96 and 19.45 kDa, which match the results obtained by EntreMed.[48] Smaller amounts of a 19.88 and 19.36 kDa bands were also apparent. Serum obtained before endostatin administration revealed small amounts of the band at

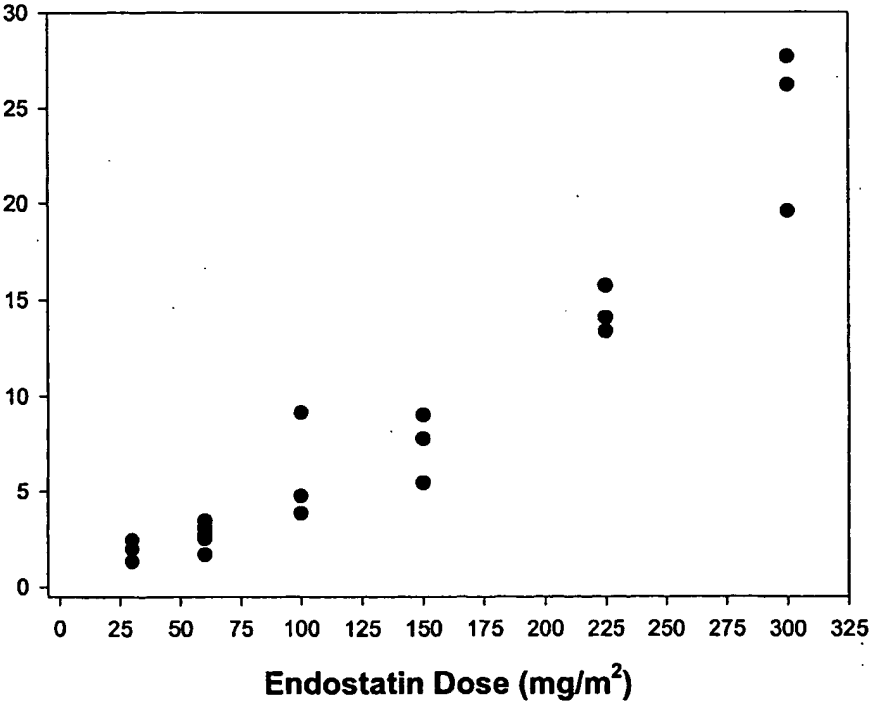

Fig 2. Endostatin total serum area under the concentration-time curve (AUC) for the first endostatin infusion (day 1 dose) as a function of endostatin dose level.

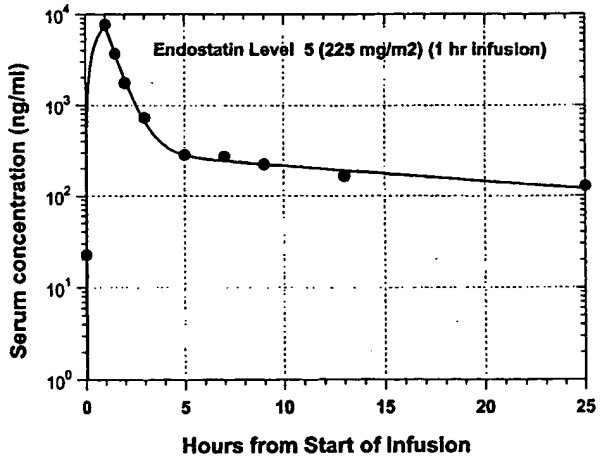

Fig 3. Serum endostatin concentration as a function of time for the first endostatin infusion (day 1 dose) in a patient receiving 225 mg/m^2 endostatin/d.

19.88 kDa, but no other significant peaks in these mass ranges. At the end of the 1-hour infusion, endostatin peaks could be readily detected. Similar results could be detected out to 6 hours after the end of the infusion (not shown). A higher relative concentration of the 19.88- and 19.36-kDa bands were detected, suggesting that some limited terminal proteolysis was occurring. No other new lower molecular weight peaks were detected by this methodology (data not shown).

Growth Factors

The effect of endostatin administration on the circulating plasma concentrations of VEGF and bFGF was examined. The effect of endostatin on circulating growth factors is unknown. bFGF concentrations obtained were low, highly variable, and showed no trend with endostatin administration (Fig 8A). Although no overall statistically significant trend in plasma VEGF with endostatin was observed (Fig 8B), several patients seemed to have depressed VEGF levels while receiving endostatin (Fig 8C). Data were similar when corrected for platelet counts (data not shown). No relationship to dose was seen.

Imaging

An extensive evaluation of tumor blood flow was attempted using dynamic CT, MRI, and ultrasound technologies. Dynamic CT has been used by other investigators to evaluate microvessel density.[51] Although no overall or dose-dependent trend was seen for patients receiving endostatin, several patients (four of 21) had changes in their time versus attenuation curves that were suggestive of a decrease in microvessel density (Fig 9). No changes in tumor blood flow or vascularity were observed from either MRI or ultrasound examination after endostatin administration. Similarly, there was no overall or dose-dependent trend in tumor glucose metabolism as evaluated by PET.

Response to Therapy

We observed no objective responses in this study. Time to tumor progression is presented in Fig 10. Several patients exhibited prolonged stable disease for periods up to 6 months. As discussed above, progressive disease in our trial was defined as a 50% increase in tumor size. Time to progression did not seem to depend on endostatin dose.

DISCUSSION

The ability of solid tumors to co-opt or engender a blood supply is central to their ability to grow and metastasize.[1,52,53] The pioneering work by Folkman et al[10,12] has defined angiogenesis as an appealing target for the development of antineoplastic compounds. The attractiveness of angiogenesis inhibitors seen preclinically is their generally low toxicity, broad efficacy, and that the target, the neovasculature endothelial cell, is genetically stable and unlikely to develop acquired resistance. Dozens of putative antiangiogenic compounds are currently undergoing clinical trials. Endostatin represents the first endogenous angiogenesis inhibitor to enter human trials. Three phase

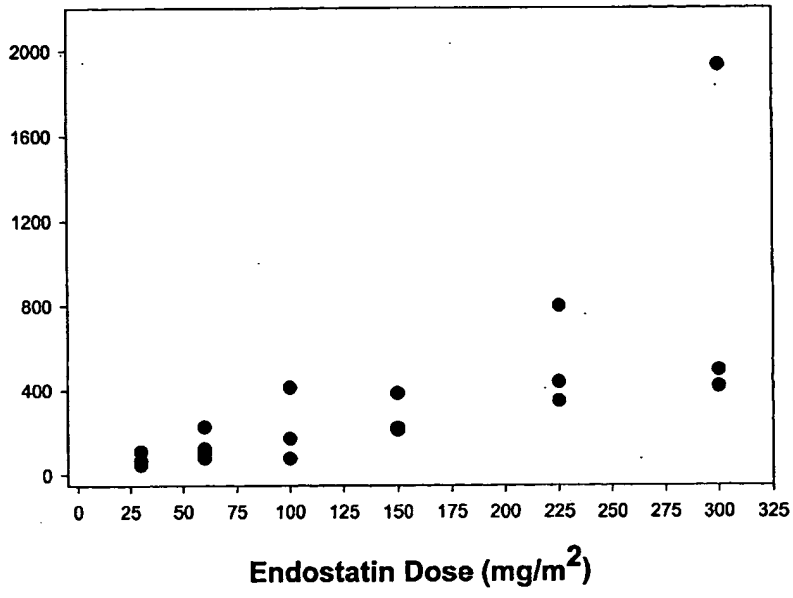

Fig 4. Trough serum endostatin concentration on day 28 as a function of endostatin dose.

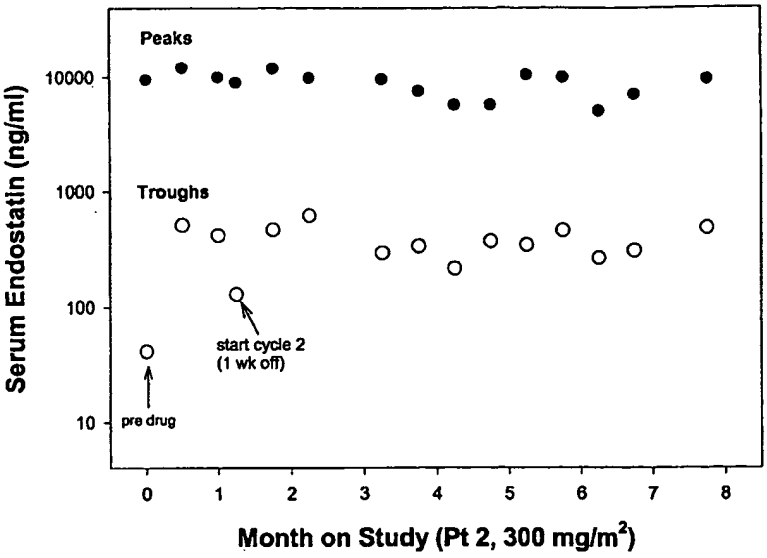

Fig 5. Serum endostatin peak and trough concentrations in a single patient receiving 300 mg/m² endostatin/d for 8 months.

I trials of recombinant human endostatin were initiated in late 1999 with considerable public attention.[54-57] The results from one of these trials is presented here. Although several thousand potential patients expressed interest in our trial, the number of patients that fulfilled eligibility criteria and were willing to make daily visits was substantially smaller.

As expected from the preclinical studies, endostatin was without significant toxicity. Indeed, the essentially complete lack of drug-related toxic effects was one of the most notable features of the three phase I trials. The absence of hematologic, gastrointestinal, renal, hepatic, neurologic, or other side effects should allow ready combination of endostatin with other agents. As noted above, several patients developed measurable titers of immunoglobulin G directed against endostatin. These antibodies were not associated with the grade 1 rashes, with other clinical sequelae, or changes in endostatin clearance. The endostatin protein in patients with antibodies still fractionated into the low molecular weight pool by FPLC, suggesting insignificant antibody binding. It is not known whether these antibodies might inhibit efficacy, but it is important to note that these relatively low-affinity antibodies were only seen in a minority of the patients.

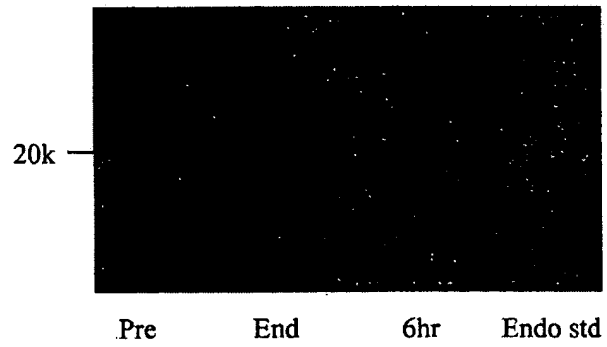

Fig 6. Endostatin Western blot assay. Plasma proteins were separated by PAGE and probed with antiendostatin antibody. Samples were drawn before endostatin infusion (Pre), at the end of the infusion (End), and 6 hours after infusion (6hr).

The clinical evaluation of angiogenesis inhibitors is challenging.[58] These agents are often nontoxic, such that definition of a maximum-tolerated dose in the phase I setting is often unrealistic. Delineation of the biologically effective dose is desirable but also difficult in that correlate assays suitable for human subjects have not been validated in preclinical models. All three groups of investigators have applied a broad spate of correlative approaches in an attempt to define a biologic effect.

We have reported on the results of our histologic evaluation of tumor and skin biopsies.[59] Paired tumor biopsies were obtained in all patients that did not have overt progression before the timing of the second biopsy (8 weeks). Eight patients had adequate pre- and posttreatment biopsies suitable for analysis. In these patients, no significant effect on microvessel density, endothelial cell proliferation, endothelial cell apoptosis, or microvessel maturity was seen. Whether the sample size or timing of the biopsies was adequate to observe a small or transient effect is questionable.

In the context of this study, we have also developed an in situ skin angiogenesis assay that has also been reported. This assay used skin wounding, with a punch biopsy that elicited brisk angiogenesis that was readily quantifiable with a second 4-mm punch biopsy 1 week later. Patients receiving endostatin had no overt differences in wound healing or vascularity. Similarly, there were no differences in endothelial cell proliferation, endothelial cell apoptosis, or blood vessel maturity. In preclinical models endostatin seems to preferentially inhibit tumor angiogenesis while sparing that involved in wound healing.[49] The simplicity and robustness of this assay may make it useful in the early clinical evaluation of other antiangiogenic agents.

No objective responses were seen in this study. As mentioned above, the best clinical effect expected with angiogenesis inhibitors may be disease stabilization. Patients receiving endostatin demonstrated no overt toxicity and in general maintained their

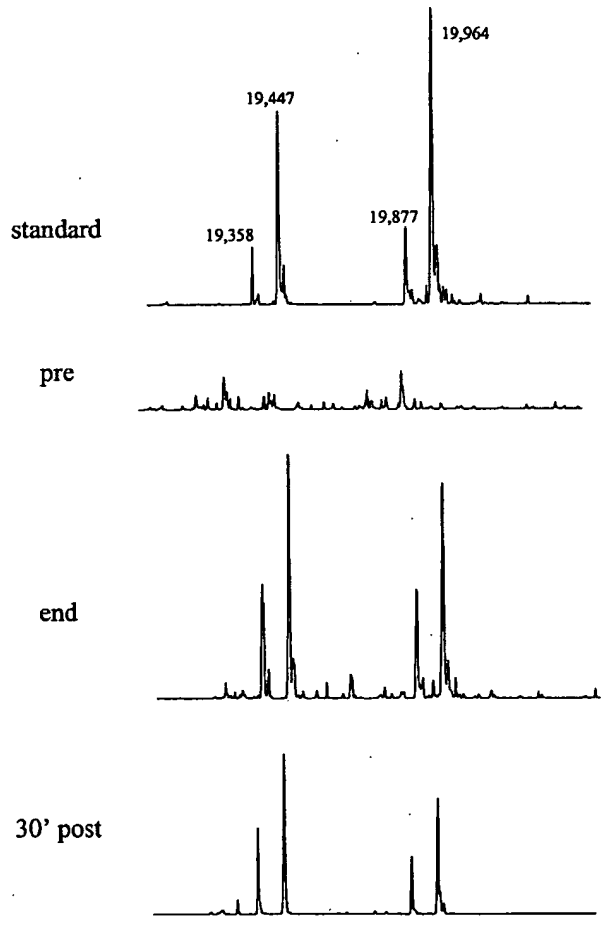

Fig 7. High-performance liquid chromatography-mass spectroscopy of FPLC fractionated proteins. Samples were drawn before endostatin infusion (pre), at the end of the infusion (end), and 30 minutes after the infusion (30' post).

performance status while enrolled in the study. Remarkably, more than 99% of the planned doses on this difficult schedule were delivered. Several patients remained enrolled in the study for prolonged periods of up to 6 months. Time to progression was not dose dependent. It should be noted again that all patients were of good performance status and up to 50% tumor growth was allowed before patients were removed from the study.

Pharmacokinetics obtained in this study were straightforward, linear with dose level, and closely mimicked those obtained in preclinical models. Pharmacokinetic parameters from the three endostatin studies have been compared and found to be consistent among the trials.[50] The levels of endostatin needed to achieve antitumor responses vary among different tumor models. The AUC achieved at the higher dose levels in our study compares favorably with AUCs of 1 to 20 μg/mL/h (2.5 to 20 mg/kg) associated with biologic activity in a number of models.[48] In vitro, endostatin inhibits microvessel proliferation at doses of around 250 ng/mL.[10]

Although the EIA assay used measures immunoreactive endostatin, it is not known whether the circulating endostatin protein retains biologic activity. Despite considerable effort, an in vitro assay for measuring endostatin antiangiogenic activity in the presence of serum or plasma was not obtainable by us or the other two groups of investigators. We examined serum endostatin by Western blot assay and did not observe the formation of lower molecular weight immunoreactive fragments. When serum endostatin was examined by mass spectroscopy, an increase in the relative proportion of the 19.88- and 19.36-kDa bands was observed. This may represent limited N- or C-terminal proteolysis of endostatin by plasma proteases. Whether these cleaved peptides retain biologic activity is unknown. The N-terminal amino acids are known to contribute to zinc binding by endostatin. Conflicting data have emerged about whether zinc binding is essential for antiangiogenic activity.[25,30,60]

The effect of endostatin administration on circulating plasma VEGF concentration has been presented here. No statistical differences in VEGF before or after endostatin administration were observed for the group as a whole, or for any of the dose levels. Several individual patients demonstrated declines in VEGF levels while enrolled in the study. The significance of this finding is unclear. It did not seem to correlate positively with time to progression in this study, although the sample size is small. To our knowledge, the effect of endostatin on circulating

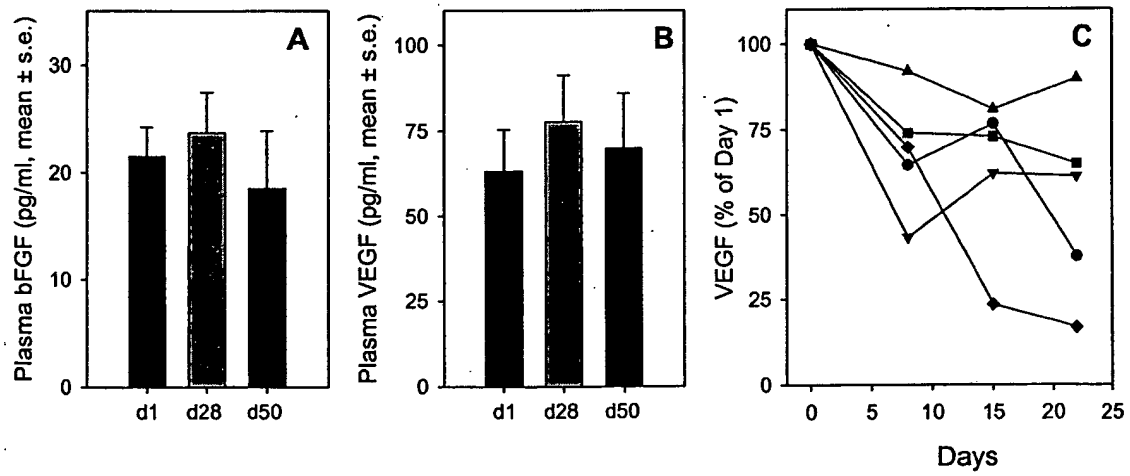

Fig 8. Circulating angiogenic growth factors in patients receiving endostatin. Levels of bFGF (A) or VEGF (B) did not significantly change after either one or two cycles of endostatin treatment. The VEGF levels in selected patients are shown in (C).

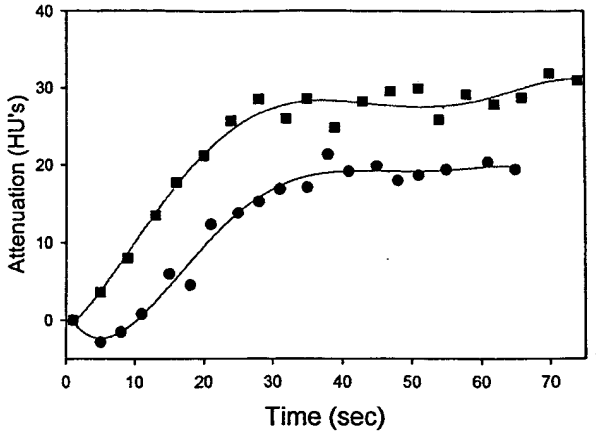

Fig 9. Dynamic CT attenuation curves. Attenuation versus time from tumor periphery at study entry (top) and after 8 weeks of endostatin administration (bottom).

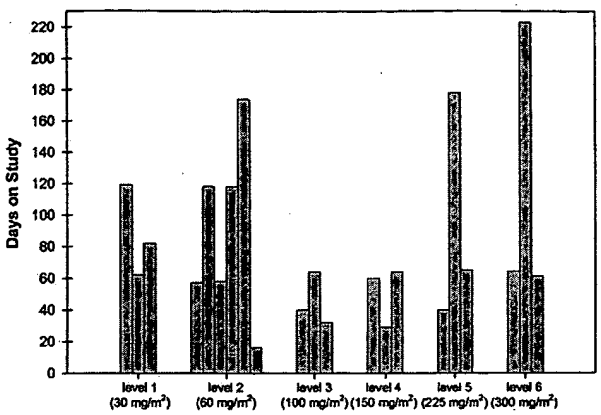

Fig 10. Time to tumor progression (50% increase) in patients receiving endostatin.

VEGF or bFGF levels has not been examined in preclinical models.

This study evaluated tumors with MRI, CT, PET, and ultrasound. Several individual patients had changes in their dynamic CT attenuation curves suggestive of decreased microvessel density. These changes were not corroborated by the biopsy data or the other imaging modalities. Changes in the dynamic CT attenuation curves were also not predictive of possible clinical benefit, such as prolonged stable disease.

The broad and dramatic activity seen with endostatin in preclinical models was not evident in our trial, although the purpose of this trial was not to establish efficacy but evaluate toxicity. There are several possible explanations why preclinical results with endostatin may be difficult to translate into the clinic. Angiogenesis in rapidly growing transplanted tumor models is likely substantially different from that in a more slowly growing human tumor with an established vasculature. Another possibility, as mentioned above, is that endostatin is undergoing limited proteolysis, denaturation, or protein-antibody binding, which renders it inactive in our patients.

The schedule of endostatin in this trial, with daily intravenous infusions, is not likely tenable for broader use. Recent evidence from animal models suggests that continuous exposure to endostatin, either through multiple subcutaneous injections or by continuous ambulatory infusion pump, may offer both greater convenience and greater efficacy.[48] A new, more concentrated formulation should allow for these alternate administrations. Future clinical trials with endostatin could employ fixed dosing because no relationship apparently exists between endostatin clearance and body surface area. The favorable toxicity profile seen with endostatin should allow for combinations with classic chemotherapeutic agents or radiation. We would further suggest that combination of endostatin with other antiangiogenic agents might hold the most promise. Angiogenesis in human tumors is likely a complex process with multiple redundant proangiogenic pathways. In vitro, higher concentrations of proangiogenic compounds such as VEGF and bFGF can overcome the inhibition of angiogenesis caused by endostatin. It is likely that turning off this essential process will require the combination of two or more antiangiogenic compounds.

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